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A NEW, AUTOMATED ASSAY FOR THE DIRECT ANALYSIS OF D-FRUCTOSE IN D-FRUCTOSE–D-GLUCOSE MIXTURES

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ABSTRACT

A new automated assay specific for D-fructose has been devised, based on the chromophore produced by dehydration of D-fructose in hydrochloric acid. The chromophore had λ_{max} at 470 (major) and 540 nm (minor). Carbohydrates not containing ketose residues do not interfere in the assay. The chromophore yields for sucrose, raffinose, and inulin were 96, 89, and 80%, respectively, of the yield from D-fructose. Advantages of the method include simplicity, inexpensive reagent, and non-interference of D-glucose. The assay was successfully applied to liquid samples taken from various stages in the industrial production of sugars.

INTRODUCTION

D-Fructose in microgram amounts in solution can be assayed manually by the resorcinol method of Yaphe and Arsenault[1] and by an automated version[2] of this procedure. D-Glucose and D-mannose interfere in this assay by ~2% when present in amounts equimolar to D-fructose[1,2]. In the automated analysis of concentrated D-glucose–D-fructose mixtures obtained from the enzymic isomerisation of concentrated (50%) solutions of D-glucose, an initial, high dilution ($\times 10^4$) is necessary and large interference from the excess D-glucose present may then be encountered, more markedly at low contents of D-fructose. Furthermore, high dilutions effected by peristaltic pumps give erratic results due to amplifications of slight fluctuations in the performance of the tubing.

Other assays[3,4] are subject to even greater interference by D-glucose. We now describe an automated assay for the direct analysis of D-fructose and D-fructose–D-glucose mixtures in which interference by equimolar D-glucose is only 0.05%.

DISCUSSION

The method utilises the ease with which a chromophore is produced from D-fructose on dehydration in hydrochloric acid solution. This chromophore is probably a mixture of oxidised derivatives[5] of the chromogens 5-(hydroxymethyl)-2-furaldehyde

2-(2-hydroxyacetyl)furan, and 5-methyl-2-furaldehyde which are initially formed[6]. The absorption spectrum for the chromophore (Fig. 1) showed maxima at 470 and 540 nm, of which the former was the superior. The exact response given by solutions of D-fructose can be varied by the time spent in the heating bath without increasing the interference due to D-glucose (Fig. 2), so that a range of D-glucose–D-fructose solutions (1–50%), as would arise from an isomerisation of D-glucose with glucose isomerase (D-glucose ketol-isomerase, E.C. 5.3.1.18), can be accurately assayed for conversion into D-fructose (100–500,000 p.p.m.). Solutions (1–100 p.p.m. of D-fructose) requiring a higher sensitivity can be assayed by the automated resorcinol procedure[2], provided the D-glucose contamination is very low.

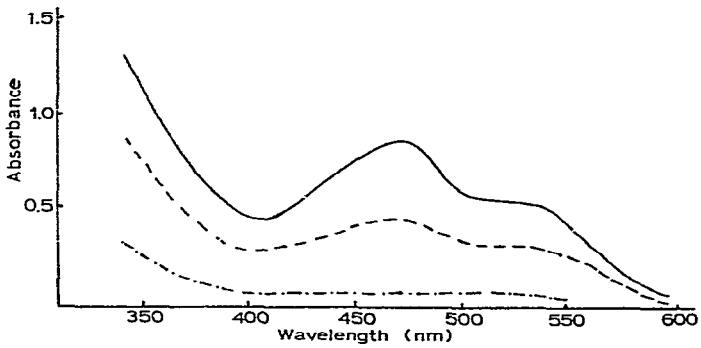

Fig. 1. Absorption spectra of the chromogens produced in the assay for D-fructose: ———, D-Fructose; — — —, sucrose; —•—•—•, L-sorbose.

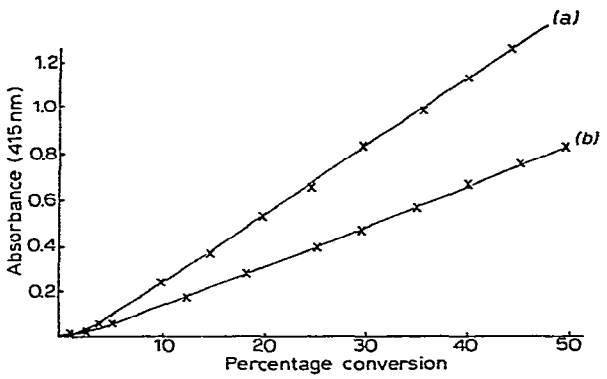

Fig. 2. Change in the assay absorbance at 415 nm with changes in the percentage conversion of (*a*) 50% D-glucose and (*b*) 10% D-glucose solutions into D-fructose. The air-flow rates and the heating rate residence times were (*a*) 3.27 ml/min, 3 min, and (*b*) 0.85 ml/min, 6 min.

Other carbohydrates, with the exception of sucrose and L-sorbose, interfere in the present assay by less than 0.2% (Table I) when present in equimolar amounts,

as does a typical buffer used in D-glucose isomerase conversions. Sucrose solutions give a slightly lower yield (96%; *i.e.*, on a molar basis calculated from 50.6% on a weight basis) of the chromophore than that expected from an equimolar mixture of D-glucose and D-fructose, probably due to incomplete hydrolysis to D-fructose under the assay conditions. The detection of sucrose in this way also showed a low interference (0.3%) by other carbohydrates (except D-fructose and L-sorbose). Since the various carbohydrates interfere to different extents at different wavelengths, the choice of wavelength is important. The assay can, therefore, also be used for sucrose in the absence of D-fructose and L-sorbose. L-Sorbose gave a relatively high (8%) molar interference in the assay; the slower reaction of L-sorbose, relative to D-fructose, to give 5-(hydroxymethyl)-2-furaldehyde has been noted before[7]. Raffinose and

TABLE I

INTERFERENCE IN THE ASSAY FOR D-FRUCTOSE[a]

Carbohydrate	*Percentage of D-fructose absorbance*		
	415 nm	*470 nm*	*540 nm*
Monosaccharides			
2-Amino-2-deoxy-D-glucose	0.00	0.00	0.00
L-Arabinose	0.20	0.04	0.00
L-Fucose	0.48	0.32	0.16
D-Fructose	100	100	100
D-Galactose	0.20	0.03	0.01
D-Galacturonic acid	3.72	1.31	1.32
D-Glucose	0.05	0.23	0.09
D-Glucurono-6,3-lactone	1.35	1.18	1.12
D-Mannose	3.41	0.50	0.09
L-Rhamnose	0.00	0.00	0.00
D-Ribose	0.00	0.00	0.00
L-Sorbose	11.15	7.52	8.09
D-Xylose	0.00	0.00	0.00
Oligosaccharides			
Cellobiose	0.00	0.00	0.00
Maltose	0.03	0.14	0.05
Raffinose	31.6	25.7	29.3
Sucrose	50.6	43.5	46.5
Polysaccharides			
Inulin	89.1	79.3	75.7
Starch	0.00	0.00	0.00
Xylan	<0.3	<0.3	<0.1
Miscellaneous			
D-Glucitol	0.00	0.00	0.00
D-Mannitol	0.00	0.00	0.00
Sodium phosphate (pH 6.8, 0.1M), 10mM in $MgCl_2$, 1mM in $CoCl_2$	0.00	0.00	0.00

[a]All carbohydrates were as 50% aqueous solutions or, if more dilute solutions had to be used, the colour equivalence was corrected.

inulin [a (1→2)-linked fructan] gave responses (88.6 and 80.2%, respectively, on a molar basis) approaching that of D-fructose, as predictable from the bound form of the D-fructose residues.

The absorption spectra of the chromophores from D-fructose and sucrose (Fig. 1, Table II) are very similar (but not identical) and quite different from that for L-sorbose.

TABLE II

RELATIVE ABSORBANCES OF THE CHROMOPHORE PRODUCED IN THE ASSAY FOR D-FRUCTOSE

Carbohydrate	*Ratio of absorbances*[a,b]		
	A^{415}/A^{470}	A^{540}/A^{470}	A^{415}/A^{540}
D-Fructose	0.58	0.58	1.00
Sucrose	0.68	0.62	1.09
L-Sorbose	0.90	0.65	1.38

[a]Absolute absorbances measured for a 20% w/v solution; [b]A^{415} = absorbance at 415 nm.

Variation of the pumping rates showed that the sensitivity of the assay (at 415 nm) could be increased thirty-fold, and interference by D-glucose under these conditions was still very low. The variation in the reproducibility of the assay was only 2% when all solutions and standard tubing used in the automating peristaltic pump were changed.

The conversion of D-glucose into D-fructose by D-glucose isomerase is becoming a widely used, commercial process. The assay described herein can, therefore, be used directly on the mixtures of D-glucose and D-fructose obtained by isomerisation of D-glucose using the free or immobilised[8,9] enzyme, giving a rapid, reproducible analysis even at low conversion rates. The assay may also be used for the determination of sucrose or raffinose in mixtures of carbohydrates (Table I) (and presumably for other carbohydrates that contain D-fructose residues). Under the conditions used, the chromophore yields for sucrose, raffinose, and inulin corresponded to 96.2, 88.6, and 80.2%, respectively, of that from D-fructose.

Application of the assay to samples taken from sugar-manufacturing processes (Table III) demonstrated its suitability not only for the monitoring of solutions of pure sugars but also for the assay of D-fructose in complex mixtures of carbohydrates and in the presence of salts. Thus, two samples (*2* and *3*) which had been shown by an independent method to contain no D-fructose (Table IV) gave negligible responses in the assay. The value of 20 mg/ml obtained for sample *1*, which contained no D-fructose, represents only a small response (equivalent to 2.0%) compared with that, for example, from 50% solutions. The response was probably due to the black colour of the original sample solution. The D-fructose value of 840 mg/ml found for sample *4* compares excellently with that (858 mg/ml) found by an independent method.

TABLE III

ANALYSIS OF COMMERCIAL SUGAR SOLUTIONS

Sample	*Equivalent fructose concentration (mg/ml)*
1 Birch hemicellulose hydrolysate, waste fraction	<20[a]
2 Purified birch hemicellulose hydrolysate	<0.7
3 Hemicellulose hydrolysate fraction after removal of xylitol	<0.2
4 Mixture of D-fructose, D-glucose, and other carbohydrates	840

[a]Coloured sample; absorbance (415 nm), 160.

TABLE IV

NEUTRAL CARBOHYDRATE ANALYSES OF COMMERCIAL SUGAR SOLUTIONS

Carbohydrate	*Concentration of carbohydrate in sample*[a]							
	Sample 1		*Sample 2*		*Sample 3*		*Sample 4*	
	%[b]	*mg/ml*[c]	%	*mg/ml*	%	*μg/ml*	%	*mg/ml*
Cellobiose	1.51	4.26	0.66	5.77	0.00	0.0	0.00	0.00
D-Ribose	10.1	22.6	1.38	9.77	9.36	691	0.00	0.00
D-Mannose	1.00	4.02	2.01	23.3	t[d]	—	s[e]	—
D-Fructose	0.00	0.00	0.00	0.00	0.00	0.0	71.4	858
L-Arabinose	11.5	24.6	2.69	17.9	6.51	424	0.00	0.00
D-Galactose	8.14	35.3	2.91	28.9	5.70	695	0.00	0.00
D-Xylose	41.4	87.8	77.5	391.2	t	—	9.33	31.5
D-Glucose	14.3	42.9	9.24	43.2	0.29	26	12.8	53.4
Miscellaneous	12.05	—	3.61	—	78.14	—	6.47	—

[a]Coding as given in Table III. [b]As a percentage of the total sugar present, based on areas under chart recorder peaks. [c]Based on standards. [d]Trace may be present. [e]Small shoulder peak not calculable.

Because the assay is simple and inexpensive, it is suitable for the continous on-line monitoring of commercial processes in the manufacture of D-fructose and sucrose. This should provide a considerable advantage over the batchwise polarimetric and gas-phase chromatographic methods currently used.

EXPERIMENTAL AND RESULTS

Materials. — The mono- and di-saccharides were commercial samples. The following liquid samples were kindly supplied by Dr. C. Aminoff (Suomen Sokeri Osakeyhtiö, Finnish Sugar Co. Ltd., Helsinki): a neutralized waste-fraction of a sulphuric acid hydrolysate (containing 8% of sodium, one half of which was present as sodium sulphate) of birch hemicellulose; purified hydrolysate of birch hemicellulose (neutralized and containing 7×10^{-3}% of sodium, as sodium sulphate); a fraction of hydrated hemicellulose hydrolysate, after removal of xylitol (neutralized

and containing $\not>0.9\%$ of sodium); a mixture of D-fructose, D-glucose, and disaccharides (containing $4 \times 10^{-3}\%$ of sodium and $2 \times 10^{-3}\%$ of calcium, predominantly as sulphates). All four samples contained phenyl mercuric acetate (0.2mM).

Solutions of mixtures of D-fructose and D-glucose were made up to represent partial isomerisation of a 50% or a 10% solution of D-glucose.

The automated assay technique. — The modular, automated equipment was set up as shown in Fig. 3. The aqueous carbohydrate solution (50% w/v) was sampled (0.015 ml/min), using a Carlo Erba peristaltic proportionating pump, and mixed with conc. hydrochloric acid (0.53 ml/min), air (3.27 ml/min), and water (0.81 ml/min). The reaction flow-stream was heated for 3 min at 85° and then cooled, and the absorbance was measured at 415, 470, and 540 nm, using Fisons Vitatron colorimeters with flow-through cells (10-mm path length). If the aqueous mixture of D-glucose and D-fructose was made up to represent partial enzymic isomerisation of a 10% solution of D-glucose, the air sampling rate was changed to 0.85 ml/min, so increasing the heating at 85° to 6 min.

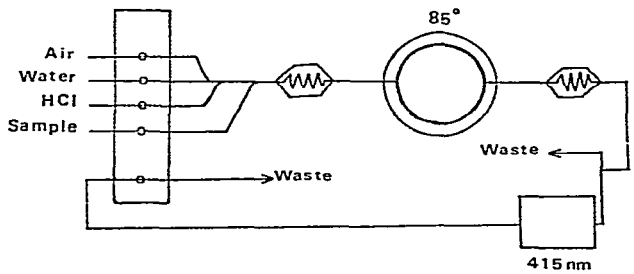

Fig. 3. Schematic diagram of the automated assay for D-fructose.

Calibration of the assay. — The assays showed linear responses against D-glucose–D-fructose content in the range 48.5–25% D-glucose and 1.5–25% D-fructose (Fig. 2*a*), or 9.7–5% D-glucose and 0.3–5% D-fructose (Fig. 2*b*). The carbohydrates tested for interference are shown in Table I.

The visible absorption spectra (Fig. 1) of the chromophore from D-fructose, L-sorbose, and sucrose are similar to that reported for the acetaldehyde-free resorcinol assay[10]. The absorption maximum of the chromophore was at 470 nm with a secondary peak at 540 nm.

In order to demonstrate an increased sensitivity of the assay with retention of realistic assay conditions, altered sampling rates were used: air, 0.42 ml/min; assay solution, 0.42 ml/min; and water (not sampled); and the heating schedule was changed to 10 min at 78°. A 1 mg/ml solution of D-fructose then gave an absorbance at 415 nm of 0.178 compared with an absorbance of 2.96 at 415 nm for a 50% (500 mg/ml) solution measured under the standard conditions. The interference by D-glucose under the more sensitive conditions was 0.17%.

The reproducibility of the assay system was tested by measuring the absorbance

at 415 nm of eight separately prepared solutions of D-glucose (40% w/v) mixed with D-fructose (10% w/v), using the different sets of standard pump tubing (but retaining the same pump rates) on different days. The average difference from the mean value was 2%.

Some applications of the assay. — Four different commercial sugar solutions were assayed in the automated system under the conditions used for 50% solutions after dilution to overcome viscosity effects. The values obtained for D-fructose-type contents, as based on a D-fructose standard, are shown in Table III. The samples were also analysed for their neutral carbohydrate components by automatic carbohydrate analysis by the borate ion-exchange chromatography/orcinol–sulphuric acid assay system, using a Jeol 6-AH carbohydrate analyser (Table IV).

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